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(71) Applicants *(for all designated States except US)*: ISIS INNOVATION LIMITED [GB/GB]; 2 South Parks Road, Oxford OX1 3UB (GB). UNIVERSITY OF BATH [GB/GB]; Claverton Down, Bath BA2 7AY (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: GALIONE, Antony [GB/GB]; Downham Cottage, Asthall, Burford, Oxfordshire OX18 4HW (GB). POTTER, Barry [GB/GB]; 95 Dovers Park, Bathford, Bath BA1 7UE (GB).

(74) Agent: PENNANT, Pyers; Stevens, Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).

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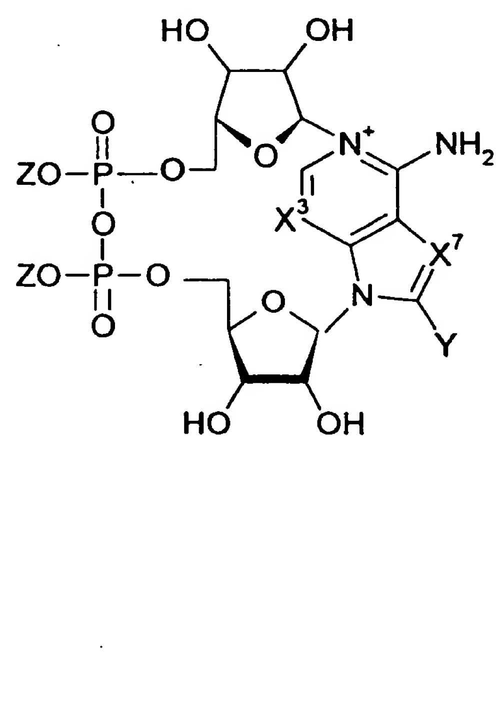
(54) Title: CYCLIC ADENOSINE DIPHOSPHATE RIBOSE ANALOGUES

(57) Abstract

A compound having formula (1) where at least one of X^3 and X^7 is CR and any remaining X^3 or X^7 is N; Y is halo, C1–C20 hydrocarbon, NR_2, OR, SR, nitro or carboxyl; R is H or C1–C20 hydrocarbon and R groups can be the same or different; and one or each Z represents H or one Z is a cageing group. The compound 7-deaza-8-bromo-cyclic adenosine 5'-diphosphate ribose has been shown to be a stable hydrolysis-resistant cADPR antagonist.



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CYCLIC ADENOSINE DIPHOSPHATE RIBOSE ANALOGUES

INTRODUCTION

Cyclic adenosine diphosphate ribose (cADPR) is a ubiquitous Ca^{2+} mobilizing metabolite of β-NAD^+ (1, 2). It is reported to mediate Ca^{2+} release via ryanodine sensitive channels in many cell types, in both animal and plant kingdoms (1, 3-6). Endogenous levels of cADPR have been detected and reported to be equally widespread (7). This finding has led to the postulation that cADPR may be the endogenous/physiological regulator of ryanodine receptors (5, 8-10).

As is the case for the more established intracellular messengers (i.e. IP_3 cAMP and cGMP), cADPR metabolising enzymes are also present which can modulate its levels (11). The synthetic activity of ADP-ribosyl cyclase and catabolic activity of cADPR hydrolase are often co-localised on the same polypeptide. In these cases. the hydrolase activity often exceeds that of cyclase (1). However. one notable exception is *Aplysia* ADP-ribosyl cyclase which is isolated and purified from soluble ovotestis extracts of the sea hare. *Aplysia californica.* The exceptionally high level of cvclase activity exhibited by this enzyme (1) has been well exploited to synthesise large quantities of cADPR. In addition, the finding that this cyclase exhibits loose substrate specificity. has allowed the development of a chemo-enzymatic synthesis of a number of cADPR analogues (12).

The first series of pharmacologically useful cADPR analogues to be synthesised were the 8-substituted analogues (13). These differ from cADPR by a substitution at the 8 position of the adenine ring. This single modification abolishes the agonistic activity of these compounds and instead produces specific competitive antagonists of

cADPR-sensitive Ca^{2+} release (13). Since its discovery, 8-NH_2-cADPR has been used successfully to demonstrate the involvement of cADPR - mediated Ca^{2+} signalling in sea urchin eggs during fertilisation (14) and NO and cGMP-induced Ca^{2+} release (15), in Purkinje neurons (16), in hippocampal synaptic plasticity, in permeabilized Jurkat T cells (6), in intestinal smooth muscle during cholecystokinin-induced contractions (5), in PC12 cells (17) and in excitation contraction coupling in cardiac myocytes (18). However, like the parent compound, cADPR, the 8-substituted analogues are prone to hydrolysis by endogenous enzymes (13). Indeed, this may explain the absence of inhibitory effect on secretogue-induced Ca^{2+} release in rat pancreatic beta-cells (19) and during induction of LTD in Purkinje neurons (16), where a role for cADPR-mediated Ca^{2+} signalling remains controversial.

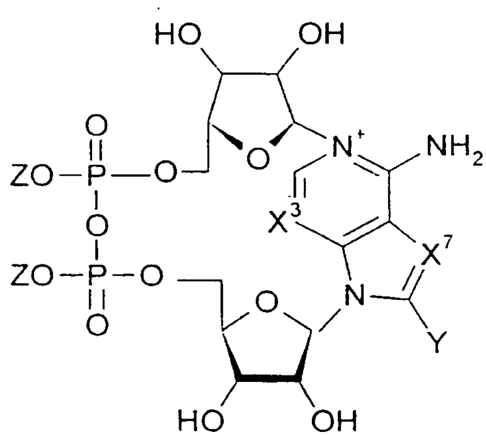
These observations underscore the need for a stable, hydrolysis-resistant cADPR antagonist. Recently, we reported on the synthesis of another analogue of cADPR, 7-deaza-cADPR. and demonstrated that it is more stable to heat-induced hydrolysis and is also a poor substrate for cADPR hydrolase (20). These changes in stability were also brought about by a single modification, that is a replacement of the 7 position nitrogen with carbon (Figure 1A). These findings then raised an intriguing question: what would be the biological activity of a compound modified at both the 7 and 8 positions of the adenosine ring? A hybrid' analogue, was successfully synthesised namely, 7-deaza-8-bromo-cADPR (Figure 1A). Its biological properties were examined and are reported herein. Our findings show that 7-deaza-8-bromo-cADPR retains both useful pharmacological properties i.e. it is a hydrolysis resistant, antagonist of cADPR-induced Ca^{2+} release. Furthermore, owing to the lipophilic nature of both the bromo- and CH- moieties, we have explored its potential as a membrane permeable analogue of cADPR, as has been established for 8-bromo-cGMP (Br-cGMP) c.f. cGMP (21). A single molecular species

exhibiting all three properties would be a very powerful pharmacological tool for investigations of cADPR-mediated Ca^{2+} signalling in intact cells. We report here that 7-deaza-8-bromo-cADPR is such a tool.

ABBREVIATIONS:

cADPR	cyclic adenosine 5'-diphosphate ribose;
ADPR	Adenosine 5'-diphosphate ribose;
IP_3	myo-inositol-(1,4,5)-trisphosphate;
cAMP	cyclic adenosine 3', 5'-monophosphate;
cGMP	cyclic guanosine 3', 5'-monophosphate;
CICR	calcium-induced calcium release
ASW	artificial sea water
IM	intracellular medium.

The invention provides compounds having the formula (1)



(1)

where at least one of X^3 and X^7 is CR and any remaining X^3 or X^7 is N,

Y is halo, C1 - C20 hydrocarbon, NR_2, OR, SR, nitro or carboxyl,

R is H or C1 - C20 hydrocarbon and R groups can be the

same or different,

and one or each Z represents H or one Z is a cageing group.

Preferably X^7 is CH and X^3 is N. The preparation of such a compound is described below. Preparation of compounds where X^7 is not CH, or where X^3 is not N can readily be achieved by known chemistry using a different starting compound 6 (see the reaction scheme below).

Y is preferably halo, particularly bromo. This is the compound whose preparation is described in detail below. Alternatively Y may be amino. Alternatively Y may be iodo, either a natural or a radioisotope such as ^{125}I. Radiolabelled compound may be useful in an assay, eg. a competitive binding assay, to screen for other compounds with more potent binding characteristics for a cADPR receptor. Compounds where Y is other than bromo may readily be prepared *inter alia* using known chemistry with a different n-succinimide derivative in reaction (i).

R is H or C1 - C20 hydrocarbon e.g. alkyl, aryl, aralkyl, alkaryl, alkenyl or alkynyl and which may be substituted or unsubstituted. Preferably R is H or C1 - C4 alkyl.

Either both groups Z represent hydrogen atoms (or negative charges) as in a conventional diphosphate system. Or one group Z may be a "cageing" group e.g. 1-(o-nitrophenyl)ethyl, whereby the compound of the invention is a caged analogue adapted to be converted into an active compound *in situ* e.g. by light irradiation. The chemistry of such caged analogues is well known, see Aarhaus R *et al*, J. Biol. Chem., 1995, 270, 7745; McCray J A and Trentham D R, Ann. Rev. Biophys., Biophys. Chem, 1989, 18, 239-270 and such compounds can be prepared by standard methods.

Although the two sugar rings have been shown as ribose for simplicity, it is envisaged that other groups may be present as in known nucleotide chemistry. Thus, on either ribose, the 5'- oxygen atom may be

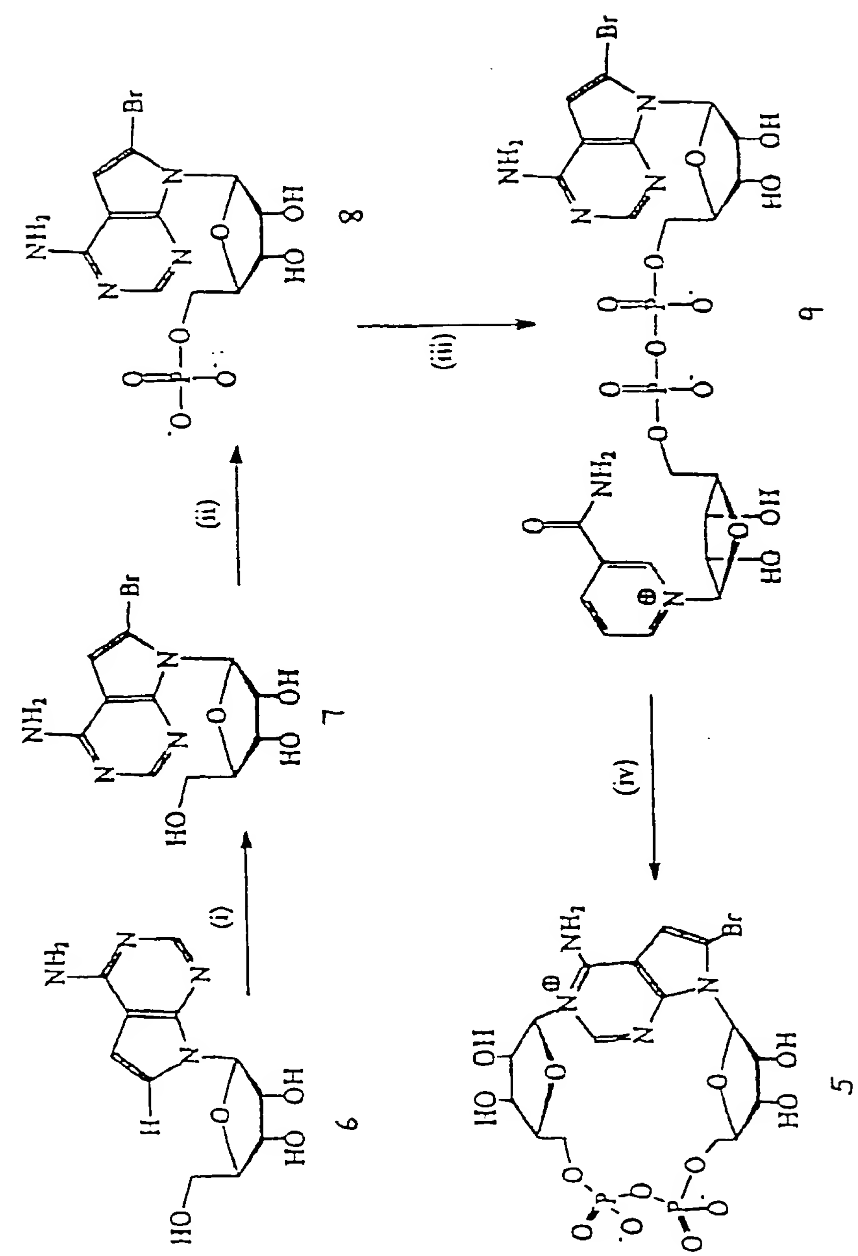
replaced by S, CHF, CF_2, SO, NR or CH_2. Also the 2'- and 3'-hydroxyl groups may be replaced by H, F, NH_2, N_3 or O-hydrocarbyl. Such compounds may be made by known chemical methods analogous to those described below.

MATERIALS AND METHODS

Chemo-enzymatic synthesis of 7-deaza-8-bromo-cADPR.

The synthesis of 7-deaza-8-bromo-cADPR. Briefly, 7-deaza-adenosine (Tubercidin) was brominated and selectively phosphorylated to yield 7-deaza-8-bromo-adenosine 5'-monophosphate (7-deaza-8-bromo-AMP). This was then coupled to nicotinamide mononucleotide (NMN) to form 7-deaza-8-bromo-β-NAD^+ as has previously been described for 7-deaza-β-NAD^+(20). The enzymatic cyclization of 7-deaza-8-bromo-NAD^+ to 7-deaza-8-bromo-cADPR was catalysed by crude *Aplysia* ADP-ribosyl cyclase and the product was purified by ion-exchange chromatography (12, 20). The extinction coefficient for 7-deaza-8-bromo-cADPR was determined by total phosphate analysis to be 10.85×10^3 $M^{-1}cm^{-1}$ (277 nm) and this value was used to determine the concentrations of 7-deaza-8-bromo-cADPR used in the rest of the study.

The reaction scheme below shows synthesis of 7-deaza-8-bromo-cADPR 5. *Reagents and Conditions:* (i) N-bromosuccinimide, K^+ acetate, DMF, 10 min, 40%; (ii) (a) $PO(Oet)_3$ 50°C, 15 min; (b) $POCl_3$ 0°C, 2h; (c) H_2O, 44%; (iii) DCC, pyridine:H_2O 4:1, 7d, 28%; (iv) 1.5mM solution in 25 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid, ADP-ribosyl cyclase, 30 min, 31%.


6
7
8
9
5
(i)
(ii)
(iii)
(iv)

Preparation of 7-Deza-8-Bromo-Adenosine (7)

To a stirred solution of dry 7 deazaadenosine (Tubercidin, 235mgs, 0.88mmol) and dry potassium acetate (185mgs, 1.88 mmol) in DMF (3.8ml) was added dropwise a solution of freshly recrystallised N-bromosuccinimide (335mgs, 1.88mmol) in DMF (1.9ml). The reaction mixture immediately turned dark red and was stirred for a further 10 min. The DMF was removed by evaporation *in vacuo* and the residue dissolved in methanol and adsorbed onto silica gel. Flash chromatography was used to purify the product with elution, using a gradient of 0-10% MeOH/$CHCl_3$. Combination of the fractions containing material with a R_f of 0.07 (10%MeOH/$CHCl_3$) yielded a yellow solid product (57) in 40% yield. This was used in the next step without further purification.

NMR δ_H (400 MHz, d_6DMS0, ppm); 3.48 (1H. m H5'; D_2O ex, ABX, J_{AB}=12.2, J_{AX}=3 9Hz) 3.66 (1H, m, H5'; D_2O ex, ABX, J_{AB}=12.2, J_{AX}=3.9Hz), 3-92 (1H, dt, J=3.9, 2.4Hz, H4'), 4.14 (1H, m, H3'; D_2O ex, dd, J=5.7, 2.4Hz), 5.13 (2H, m, H2' and 3'OH; D_2O ex, dd, J=6.8, 5.7Hz, 1H), 5.31 (1H, d, J=6.6Hz, D_2O ex, 2'0H), 5.70 (1H, dd, J=5.6, 3.8Hz D_2O ex, 5'OH), 5.84 (1H, d, J=6.8Hz) H1'), 6.80 (1H. s, H7), 7.29 (2H, br s. D_2O ex, NH_2), 8.02 (1H, s, H2).

NMR δ_C (100.4 MHz, d_6DMSO, ppm); 62.7 (C5'), 71.3 (C3'), 71.6 (C2'), 86.5 (C4'), 90.4 (C1'), 103.3 (C7), 104.3 (C5), 109.7 (C8), 149.9 (C4), 151 9 (C2), 156.9 (C6),

m/z (FAB^+) 345, 347 (M^++1), 213, 215 (M^++1-ribose).

Preparation of 7-Deaza-8-Bromo~Adenosine 5'-Monophosphate (8)

To a cooled solution of dry 7-deaza-8-bromo-adenosine (100 mg 290 μmol) suspended in triethyl phosphate (2ml) was added $POCl_3$ (100 μl, 500 μmol) and the reaction was monitored by HPLC (starting material 1.74min, product 2.15min). After 90min cooled 1:9 pyridine: water (10ml) was added to the above followed by stirring for a

further 30min. This product was purified by ion exchange chromatography and the fractions containing the product, which eluted between 310 and 370mM TEAB, were pooled evaporated *in vacuo* to give the pure product in 44% yield.

NMR δ_H (400 MHz, D_2O) 4.08-4.15 (3H. m, H4' and H5'), 4.53 (1H, dd, J=5.7, 5.8Hz, H3'), 5 07 (1H, dd, J=5.3, 5.8Hz, H2'), 6.04 (1H, d, J=5.3Hz, H1'), 6.42 (1H, s, H7), 7.92 (1H, s, H2),

δ_P (161.7MHz, D_2O); 2.35ppm, broadens when proton coupled,

δ_C (100.4MHz, D_2O); 65.2 (d. J_{CP}=3.7Hz, C5'), 70.3 (C3'), 72.1 (C2'), 83.6 (d, J_{CP}=7.4Hz, C4'), 89.8 (C1'), 104.0 (C5), 105.2 (C7), 110.4 (C8), 149.9 (C4). 150.0 (C2), 154.5 (C6),

UV: λ_{max}276 nm, $\varepsilon=13.34\times10^{3}\ ^{-1}cm^{-1.}$

HPLC retention time: product 2.151min,

m/z (FAB^+) 425, 427 (M^++l); (FAB^-) 423, 425 (M^+-2), 341 (M^+-Br-3).

Preparation of 7-Deaza-8-Bromo-Nicotinamide Adenine Dinucleotide (7-Deaza-8-Bromo-NAD$^-$) (9)

To a solution of 7-deaza-8-bromo-adenosine 5'-monophosphate (94µmol) and NMN (20mg, 60µmol) in MilliQ water (1ml) was added pyridine (4ml) and an excess of DCC (0.5g) and the reaction was stirred at room temperature for 7 days. The reaction was quenched by the addition of MilliQ water (50ml), the mixture kept at 4°C for 2h, filtered and extracted with $CHCl_3$ (3x20ml). The material was purified by ion exchange chromatography and the fractions containing the product, which eluted between 90 and 120mM TEAB, were combined, the solvent removed *in vacuo* and excess TEAB removed by coevaporation with MeOH to yield the title compound in 27.7% yield.

NMR: δ_H (400MHz, D_2O); 4.03-4.32 (8H, m, H_N2', H_N3', H_A4', H_N4', $2H_A5'$ $2H_N5'$), 4.56 (1H, dd, J=5.8, 5-3Hz, H_A3') 5 16 (1H, dd, J=5.9, 5.8HZ, H_A2'), 5 99 (1H, d, J=4.4Hz, H_N1'), 6.06 (1H, d, J=5.9HZ, H_A1'), 6.60 (1H, s, H_A7), 8.05 (1H, s. H_A2), 8.14 (1H, dd, J=8.3, 6.4HZ, H_N5), 8.73 (1H, d, J=8.3Hz, H_N4), 9.09 (1H. d, J=6.4HZ, H_N6), 9.25 (1H, s, H_N2).

δ_P (161 7MHZ, D_2O); -10.5 -10.9ppm (2d, J_{PP}= 20.1HZ),

δ_C (100.4 Hz, D_2O); 65 7 (d, J=3.7Hz, C_A5'), 66.5 (C_N5') 70.0 (C_A3') 71 4 (C_N3'), 7 1.9 (C_A2'), 78.5 (C_N2'), 83.5 (d, J=9.2Hz, C_A4'), 87.8 (d, J=9.2Hz, C_N4'), 89.8 (C_A1'), 100.9 (C_N1'), 104.3 (C_A5), 105.0 (C_A7), 110.5 (C_A8), 129 6 (C_N4), 134.2 (C_N3), 140.6 (C_N5), 143.1 (C_N6), 146.3 (C_N2), 150.4 (C_A2), 150.7 (C_A4), 155.5 (C_A6), 166.1 (CO),

UV: λ_{max}=270nm, ε=13.3x10^{3}M^{-1}cm^{-1},

HPLC retention time 2.1 6min,

m/z (electrospray$^+$) 741, 743 (M$^+$+I); (electrospray$^-$) 739, 741 (M$^+$-1).

Preparation of 7-Deaza-8-Bromo Cyclic Adenosine Diphosphate Ribose (7-Deaza-8-bromo-cADPR) (5)

7-Deaza-8-bromo NAD$^+$ (triethylammonium salt, 12μmol)) was cyclised as described and the product 7-deaza-8-bromo-cADPR which eluted between 130 and I150mM TEAB, was evaporated to dryness *in vacuo* keeping the water bath temperature below 30°C. Excess TEAB was removed by evaporation with MeOH and the cyclic compound was isolated as the glassy triethylammonium salt in 31% yield as quantified by UV and stored at -70°C.

NMR δ_H (400MHz, D_2O); 3.89-3.93 (1H, m, H_A5'), 3.98-4.03 (1H, m, H_R5'), 4 1-4.20 (1H, m, H_A4'),4.23-4.27 (1H, m, H_R5'), 4.30-4.40 (2H, m, H_A5', H_R3'), 5.44 (1H, dd, J=5.2, 6.1Hz, H_A2'), 5.97 (1H, d, J=4.0Hz, H_R1'), 6.03 (1H, d, J=6.1Hz, H_A1'). 6 91 (1H, s, H_A7), 8 75 (1 H, s, H_A2), all other protons are obscured by the water peak at 4 8ppm

δ_P (161. 7MHz, D_2O); -10.8, -11.6ppm (2d, J_{PP}=18HZ),
UV: λ_{max} =277Dm, ε=10-85x$10^3M^{-1}Cm^{-1}$,
HPLC retention time 4.8min,
m/z (electrospray$^+$) 619, 621 (M^++1); (electrospray$^-$) 617, 619 (M^+-1), 79, 81 (Br^-).

***In vitro* Ca^{2+} release assays**

In vitro Ca^{2+} release assays were performed on sea urchin egg homogenates (2.5%, v/v) prepared from unfertilised *Lytechinus pictus* eggs according to the method of Clapper and Lee (22) with modifications as previously described (23). Extramicrosomal Ca^{2+} was thus measured by monitoring changes in fluo-3 (3 μm) fluorescence (excitation 490 nm and emission 530 nm) in a Perkin-Elmer LS-SOB fluorimeter. All additions (not exceeding 5 μl) were made to cuvettes (containing 500 μl homogenate) in intracellular medium (1M) (containing 10 μm EGTA).

***Intracellular free* Ca^{2+} *measurements in intact* eggs**

Ca^{2+} imaging of intact cells was performed using unfertilised *Lytechinus pictus* eggs micro-injected with 2 μM fura-2 and 250 μg/ml heparin as previously described (23, 24).

Materials

Lytechinus pictus sea urchins were from Marinus Inc. Long Beach, CA. USA. Fluo-3 and Fura 2 was purchased from Cambridge Bioscience (Molecular Probes). All other chemicals were from Sigma Inc. (London). Cyclic ADP-ribose, 8-bromo-cADPR and 7 deaza-cADPR were synthesised as previously described (12, 20).

RESULTS & DISCUSSION

Since the elucidation of the structure of cADPR (25, 26) and

the development of a chemoenzymatic synthesis for cADPR and its analogues (12), interest has mounted concerning the structure-function relationships between the ligand and its endogenous receptor(s). To further aid these diagnostic studies, we synthesised 7-deaza-8-bromo-cADPR. a novel analogue of cADPR. Figure 1A shows the chemical structure of 7-deaza-8-bromo-cADPR as compared with that of cADPR and two previously reported analogues 8-bromo-cADPR, a specific cADPR antagonist (10) and 7-deaza cADPR, a hydrolysis resistant, partial agonist (20). Whereas the latter two compounds have a single modification made at either the 7 or 8 position of the adenosine ring, 7-deaza-8-bromo-cADPR possesses both these modifications. The effect of each modification on the Ca^{2+} mobilizing (agonistic) ability was first tested using sea urchin egg homogenates (2.5%). Figure 1B shows the Ca^{2+} releasing action of 2 μM applications of 7-deaza-8-bromo-cADPR and related cyclic nucleotides. Unlike the agonists cADPR and 7-deaza cADPR, 7-deaza-8-bromo-cADPR did not induce Ca^{2+} release from sea urchin egg microsomes, even at concentrations up to 20μM (a supra-maximal agonist concentration). In this respect 7-deaza-8-bromocADPR resembles the antagonist 8-bromo-cADPR.

Whether 7-deaza-8-bromo-cADPR, like 8-bromo-cADPR, was also an antagonist of cADPR-sensitive Ca^{2+} release was investigated next. Figure 2 shows that this was indeed the case. Sea urchin egg homogenates pre-treated with either 8-bromo-cADPR or 7-deaza-8-bromo-cADPR were markedly less responsive to 100 nM cADPR (Figure 2A). These inhibitory actions were dependent on the antagonist concentration (Figure 2B). Both 8-bromo-cADPR and 7-deaza-8-bromo-cADPR exhibited similar inhibition potencies with comparable IC_{50} values (IC_{50} = 0.97 ± 0.04μm (S.E. n=3) for 8-bromo-cADPR: IC_{50}= 0.73 ± 0.05 μM (S.E. n=3) for 7-deaza-8-bromo-cADPR.).

Lower concentrations (31 mM) of either antagonist were also

significant in preventing Ca^{2+} release by 100 nM cADPR which did not exceed 85% of control values. Whether this is due to the presence of more than one population of receptors which exhibit different binding affinities and/or sensitising properties remains to be investigated. Nonetheless, both 8-substituted analogues appeared to behave similarly with respect to these actions on the cADPR-induced Ca^{2+} release channel in sea urchin egg homogenates. As previous studies have shown that 8-substituted cADPR analogue (7,13, [new 14] and 7-deaza-cADPR (20) are able to displace cADPR binding, it is likely that 7-deaza-8-bromo-cADPR also interacts at the cADPR receptor in the same specific manner.

Since a modification on the 8-position does not alter the stability of the molecule (13) but a substitution of N^7 with a carbon has been shown to render the cyclic compound more resistant to hydrolysis (20), we investigated whether, 7-deaza-8-bromo-cADPR could differ from 8-bromo-cADPR, but resemble 7-deaza-cADPR in this respect. We subjected standard solutions of both antagonists to heat-induced hydrolysis. This treatment has previously been shown to strip unstable cyclic compounds such as cADPR and 8 NH_2-cADPR, of their biological activity (13, 18, 20). Figure 3 shows the effect of heat treatment on the antagonistic actions of both 7-deaza-8-bromo-cADPR and 8-bromo-cADPR. Whereas 8-bromo-cADPR is stripped of its antagonistic activity, 7-deaza-8-bromo-cADPR remained an effective antagonist of cADPR-induced Ca^{2+} release (Figure 3B compared to A). HPLC analysis of the same samples confirmed that this loss of activity was due to degradation of 8-bromo-cADPR whilst 7-deaza-8-bromo-cADPR remained intact (data not shown). These data are in line with the chemical stability of 7-deaza adenosine *c.f.* adenosine (27) and suggests that 7-deaza-8-bromo-cADPR is the first stable cADPR antagonist. This new compound therefore has potential for application in intact cells/tissues such as Jurkat T cells and neuronal tissue which express high hydrolase activities but may still have a functional cADPR-

mediated Ca^{2+} signalling pathway (1).

When these antagonists were tested for resistance to enzyme mediated hydrolysis (by cADPR hydrolases) a similar resistance emerged. Egg homogenates (2.5%. containing fluo-3), were incubated overnight (at 17°C) with 20 μM 8-bromo-cADPR or 7-deaza-8-bromo-cADPR. Samples (50 μl) were then taken and tested for antagonistic activity on cADPR-induced Ca^{2+} release (as described for figure 2 but where the cuvette concentration of the antagonists was initially 2 μM). Whereas the antagonistic activity of 8-bromo-cADPR, in response to cADPR (100 nM), had dramatically reduced following the prolonged incubation with *L. pictus* cADPR hydrolase (65.8 ± 8.3% of control cADPR-induced Ca^{2+} release, n=3), the levels of 7-deaza-8-bromo-cADPR remained high thereby producing a greater antagonistic effect on cADPR-induced Ca^{2+} release (28.5 ± 11.4% of control cADPR-induced Ca^{2+} release, n=3). This was also confirmed by HPLC analysis of the same samples (data not shown).

It has been demonstrated that the presence of a lipophilic bromine moiety in cGMP affords greater membrane permeability to Br-cGMP (21) and the replacement of a nitrogen with CH- group also offers greater hydrophobicity (28). Therefore, the novel cADPR analogue, 7-deaza-8-bromo-cADPR, should have greater hydrophobic character than any previously synthesised. We investigated this by testing the effect of extracellular applications of 7-deaza-8-bromo-cADPR on fertilisation-induced Ca^{2+} mobilisation in intact sea urchin eggs. Eggs were co-injected with the $1P_3$ receptor antagonist, heparin (250 μg/ml) and Ca^{2+} sensitive fluorochrome fura-2 (2 μM). Figure 4A shows that upon sperm addition to control heparinized eggs, a propagating Ca^{2+} wave was produced (Figure 4A, open squares). This Ca^{2+} wave had an average amplitude of 1705 ± 119 nM Ca^{2+} (S.E.M, n = 11) and took 41.9 ± 5.8 s (S.E.M, n = 11) to reach this peak. These data are consistent with previously reported

observations of sperm-induced Ca^{2+} mobilization from IP_3-insensitive Ca^{2+} stores (14, 29). When heparinized eggs were pre-incubated (5 minutes) with bathing solution containing 7-deaza-8-bromo-cADPR (100 μM). the sperm-induced intracellular Ca^{2+} transients were completely abolished (figure 4A, filled circles) suggesting inhibition of the redundant cADPR-sensitive Ca^{2+} release mechanism (14., 29). In contrast. when 8-bromo-cADPR was present in the bathing medium, a Ca^{2+} transient was still observed, albeit dramatically reduced (figure 4A, filled triangles). The wave property of the Ca^{2+} response was also abolished by both treatments where the Ca^{2+} rises occurred slowly (if at all) and over the whole egg. Although the inhibition of fertilisation-induced Ca^{2+} transients was dependent on antagonist concentration (figure 4B. filled symbols), 7-deaza-8-bromo-cADPR appeared to be the more effective antagonist. Neither antagonist released Ca^{2+} in the eggs during the 5 minute pre-incubation period which is consistent with the absence of agonistic activity observed *In* vitro (figure 1B).

Since intracellular Ca^{2+} mobilization is a pre-requisite for the cortical reaction (30, 31), we also monitored the formation of activation envelopes following fecundation. Treatment of eggs with either 7-deaza-8-bromo-cADPR or 8-bromo-cADPR in the bathing medium, was found to prevent the cortical reaction (in heparinized eggs) in a concentration dependent manner (Figure 4B, open symbols). This is in keeping with previous reports which demonstrate inhibition of the cortical reaction only once both redundant mechanisms have been blocked (14, 29). Eggs that were treated with antagonist only (i.e. not micro-injected with heparin), were also scored for activation envelopes in the same experiments. These consistently showed >95% activation. This suggested that neither 7-deaza-8-bromo-cADPR nor 8-bromo-cADPR acted as spermicides, rather they are able to permeate the sea urchin egg plasma membrane and specifically compete for endogenous cADPR-binding sites and inhibit

agonist-induced Ca^{2+} mobilisation. Presumably, since the net charge on 7-deaza-8-bromo-cADPR is only -1 at physiological pH (figure 1A), this compares well with 8-bromo-cGMP which has the same net charge and where 8-substitution confers membrane permeability. This property now eliminates the need for potentially disruptive protocols such cell permeabilization or micro-injection as methods for introducing cADPR antagonists into whole cells. This advancement should greatly aid investigations of the role of c.ADPR in physiological responses to extracellular stimuli.

In conclusion. we have demonstrated that, unlike 8-bromo-cADPR. 7-deaza-8-bromo-cADPR is a stable hydrolysis-resistant cADPR antagonist. This is the first report of such a compound. In addition, we have exploited the lipophilic nature of the bromo-moiety to produce a compound that is also sufficiently membrane permeable. In all this makes 7-deaza-8-bromo-cADPR a very powerful pharmacological tool for investigations of cADPR-mediated Ca^{2+} signalling in intact cells.

REFERENCES

1. Lee, H.C., Galione. A. and Walseth, T.F. (1994) in *Vitamins and Hormones* (Litwack, G., ed.), Vol. 48. pp. 199-257, Academic Press. London.

2. Galione, A. and Sethi, J. (1996) in *Biochemistry of Smooth Muscle Contraction* (Barany, M., ed.), pp. 295-305, Academic Press, London.

3. Hua, S.Y., Tokimasa. T., Takasawa, S., Furuya, Y., Nohmi. M., Okamoto. H. and Kuba, K. (1994) *Neuron* **12**, 1073- 1079.

4. Allen, G.J., Muir, S.R. and Sanders, D. (1995) *Science* **268**, 735-737.

5. Kuemmerle. J.F. and Makhlouf, G.M. (1995) J: *Biol. Chem.* **270**. 25488-25494.

6. Guse. A.H., DaSilva. C.P., Emmrich, F., Ashamu. G.A., Porter. B.V.L. and Mayr. G.W. (1995) J. Immunol. **155,** 3353-3359.

7. Walseth, T.F., Aarhus. R., Zeleznikar, R. and Lee. H.C. (1991) *Biochim. Biophys. Acta* **1094**, 113-120.

8. Galione, A. and Summerhill. R.J. (1996) in *Ryanodine Receptors* (Sorrentino. V., ed.), pp. 51-70. CRC Press Inc., London.

9. Sitsapesan, R., McGarry. S.J. and Williams, A.J. (1995) *Trends Pharmacol. Sci.* **16**. 386-391.

10. Meszaros, L.G., Bak. J. and Chu, A. (1993) *Nature* **364**, 76-79.

11. Rusinko, N. and Lee, H.C. (1989) J: *Biol. Chem.* **264**, 11725-11731.

12. Ashamu, G.A., Galione. A. and Potter, B.V.L. (1995) J *Chem. Soc., Chem. Commun.,* 1359-1360.

13. Walseth, T.F. and Lee, H.C. (1993) *Biochim. Biophys. Acta.* **1178,** 235-242.

14. Lee, H.C., Aarhus, R. and Walseth, T.F. (1993) *Science* **261**, 352-355.

15. Willmott, N., Sethi, J.K., Walseth, T.F., Lee, H.C., White, A.M. and Galione, A. (1996) J: *Biol. Chem.* **271**, 3699-3705.

16. Linden, D.J., Dawson, T.M. and Dawson, V.L. (1995) *J Neurosci* **15**, 5098-5105.

17. Clementi, E., Riccio, M., Sciorati, C., Nistico, G. and Meldolesi, J. (1996) J *Biol.Chem.* **271**, 17739-17745.

18. Rakovic. S., Galione. A., Ashamu, G.A., Potter, B.V.L. and Terrar. D.A. (1996) *Curr. Biol.* **6,** 989-996.

19. Willmott, N.J., Galione, A. and Smith. P.A. (1995) *Cell Calcium* **18**, 411-419.

20. Bailey, V.C., Sethi, J.K., Fortt, S.M., Galione, A. and Potter, B.V.L. (1997) *Chem.& Biol.*, **4**, 51-61.

21. Butt, E., Nolte. C., Schulz, S., Beltman, J., Beavo, J.A., Jastorff. B. and Walter, U. (1992) *Biochem. Pharmacol.* **43**, 2591-2600.

22. Clapper, D.L., Walseth, T.F., Dargie. P.J. and Lee, H.C. (1987) *J Biol. Chem.* **262**, 9561 -9568.

23. Sethi. J.K., Empson, R.M. and Galione, A. (1996) *Biochem.* J. **319**, 613-617.

24. Genazzani. A.A., Empson. R.,M. and Galione. A. (1996) J. *Biol. Chem.* **271**, 11599-11602.

25. Kim, H., Jacobson, E.L. and Jacobson. M.K. (1993) *Biochem. Biophvs. Res. Commun.* **194,** 1143-1147.

26. Lee, H.C., Walseth, T.F., Bratt. G.T., Hayes, R.N. and Clapper. D.L. (1989) J *Biol. Chem.* **264**, 1608- 1615.

27. Pike. J.E., Slechta. L. and Wilev. P.F. *(1964) J.Heterocyclic Chem.* **1**, 109-161.

28. Ji, L.N., Corfu, N.A. and Sigel. H. (1991)J. *Chem. Soc.-Dalton Trans.,* 1367-1375.

29. Galione, A., McDougall. A., Busa, W.B., Willmott, N., Gillot, I. and Whitaker, M. (1993) *Science* **261**, 348-352.

30. Sasaki, H. (1984) *Dev. Biol.* **101**, 125-135.

31. Sardet, C. and Chang, P. (1987) *Cell Differ.* **21**, 1-19.

FIGURE LEGENDS

Figure 1: (A) Structural formulae of cADPR 7-deaza-cADPR, 8-bromo-cADPR and 7-deaza-8-bromo-cADPR. Modifications were made to cADPR at the 7 and 8 positions of the adenosine ring. The N^7 nitrogen atom is replaced with a carbon (and associated proton) to form 7-deaza cADPR whilst 8-bromo-cADPR is formed by substituting the hydrogen on the C-8 position with a bromine atom. 7-Deaza-8-bromo-cADPR has both these modifications. **(B)** Calcium releasing action of cADPR, 7-deaza-cADPR, 8-bromo-cADPR and 7-deaza-8-bromo-cADPR in sea urchin egg

homogenates. *Lytechinus pictus* egg homogenates (2.5%, v/v) containing the Ca^{2+} sensitive dye. fluo-3 (3 μM) was prepared as described in Materials and Methods. Aliquots (500 μl) of these were challenged with 5 μl of cyclic compounds to give a final cuvette concentration of 2 μM. The Ca^{2+} release profiles observed are shown and are representative of three separate experiments.

Figure 2: Concentration dependent inhibition of cADPR-induced Ca^{2+} release by 8-bromo-cADPR and 7-deaza-8-bromo-cADPR.

Lytechinus pictus egg homogenates (2.5 %, v/v) containing the Ca^{2+} sensitive dye, fluo-3 (3 μM) were prepared as described in Materials and Methods. Their sensitivity to cADPR-induced Ca^{2+} release was used to test the antagonistic action of 8-bromo-cADPR **(A)** as compared to that of 7-deaza-8-bromo-cADPR **(B)**. This was done by pre-treating homogenates with increasing concentrations of antagonist (in 5μl Glu IM + EGTA), 3 minutes prior to challenge with 100 nM cADPR. Representative traces are shown in (A) and (B) where the addition artefact has been removed for clarity (gap in traces). The amount of Ca^{2+} released by cADPR application was determined and expressed as a percentage of control cADPR release, i.e. the amount of Ca^{2+} released in homogenates treated with vehicle. The inhibition curves that were obtained for each antagonist are shown in **(C)**. Each value represents the mean ± SE of triplicates. The IC_{50} values were estimated to 0.97 ± 0.04 μM for 8-bromo-cADPR and 0.73 ± 0.05 μM for 7-deaza-8-bromo-cADPR (S.E. n=3).

Figure 3: Effect of heat treatment on antagonist activity of 8-bromo-cADPR and 7-deaza-8-bromo-cADPR.

Experimental conditions were as for figure 2. Complete inhibition of cADPR-induced release was seen when homogenates were pre-treated with 10 μM of either 8-bromo-cADPR or 7-deaza-8-bromo-

cADPR **(A)**. The stock solutions (1 mM) of either 8-bromo-cADPR or 7-deaza-8-bromo-cADPR were also incubated at 85°C for 90 min in a water bath. After heat-induced hydrolysis, the antagonistic action of 8-bromo-cADPR was abolished **(B)**. In contrast. heat-treated 7-deaza-8-bromo-cADPR remained an effective antagonist to cADPR-induced Ca^{2+} release. Fluorimetric traces are representative of three similar experiments.

Figure 4 . Antagonistic actions of extracellularly applied 7-deaza-8-bromo-cADPR and 8-bromo-cADPR on fertilisation-induced Ca^{2+} transients in intact sea urchin eggs.

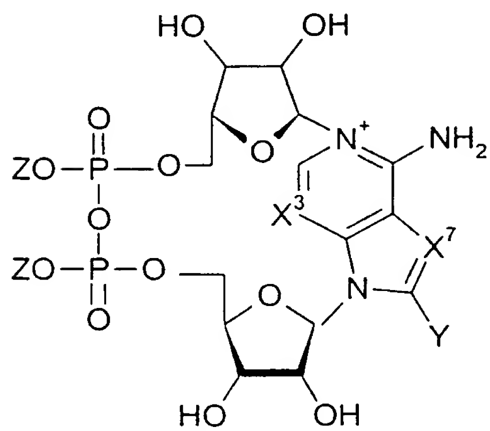
(A). Control response (open squares) showing a typical fertilisation-induced Ca^{2+} transient in intact *L. pictus* eggs pre-injected with heparin and fura-2 (final concentrations in egg were approximately 250 μg/ml and 2 μM respectively). Sperm were added at time = 0. Despite the presence of heparin, an IP_3 receptor antagonist, the wave properties of the sperm-induced Ca^{2+} rise were intact and activation envelopes were formed (14, 29). The peak Ca^{2+} rise following the addition of sperm was 1705 ± 119 nM Ca^{2+} (SEM, n=11). Addition of 100 μM 7-deaza-8-bromo-cADPR in the bathing medium (5 minutes prior to sperm addition), completely abolished the fertilisation-induced Ca^{2+} response (filled circles). However, the same concentration, 100 μM, of 8-bromo-cADPR only reduced the Ca^{2+} transient (filled triangles). The reduction of the fertilisation transient with 100 μM 8-bromo-cADPR was similar to that seen using 50 μM 7-deaza-8-bromo-cADPR (988 ± 81 nM Ca^{2+}; SEM, n=6) supporting the greater effectiveness of the 7-deaza-8-bromo-cADPR compound. Neither antagonist mobilized Ca^{2+} in the eggs during the 5 minute pre-incubation period.

(B). Dose-dependent action of 7-deaza-8-bromo-cADPR and 8-bromo-cADPR on peak fertilisation-induced Ca^{2+} rise and egg activation.

For each concentration of 7-deaza-8-bromo-cADPR (circles) and 8-bromo-cADPR (triangles), 4-9 eggs in a single dish were co-injected with heparin (250 μg/ml) and Fura (2 μM). Following incubation in artificial sea water containing 7-deaza-8-bromo-cADPR or 8-bromo-cADPR for 5 minutes sperm were added and the maximum change in intracellular free Ca^{2+} (primary Y-axis, filled symbols) was monitored on one of the pre-injected eggs. Following recovery of the Ca^{2+} transient we scored the presence or absence of an activation envelope in all injected eggs. The results (secondary Y-axis, open symbols) show that in heparinized eggs bathed in artificial sea water containing either 7-deaza-8-bromo-cADPR or 8-bromo-cADPR, there was a dose dependent reduction in the % of the eggs activated. 7-deaza-8-bromo-cADPR appeared to be more effective at all concentrations. Remaining non-heparinized eggs were all consistently activated, indicating that sperm activity was unaffected by increasing concentrations of either antagonist.

CLAIMS

1. A compound having the formula (1)



(1)

where at least one of X^3 and X^7 is CR and any remaining X^3 or X^7 is N,

Y is halo, C1 - C20 hydrocarbon, NR_2, OR, SR, nitro or carboxyl,

R is H or C1 - C20 hydrocarbon and R groups can be the same or different,

and one or each Z represents H or one Z is a cageing group.

2. 7-Deaza-8-bromo-cyclic adenosine 5'-diphosphate ribose.

3. A method of screening for a compound which binds to a cADPR receptor, which method comprises performing a competitive binding assay in which the compound is caused to compete with a compound according to claim 1 or claim 2 for binding to the cADPR receptor.

Fig.1.

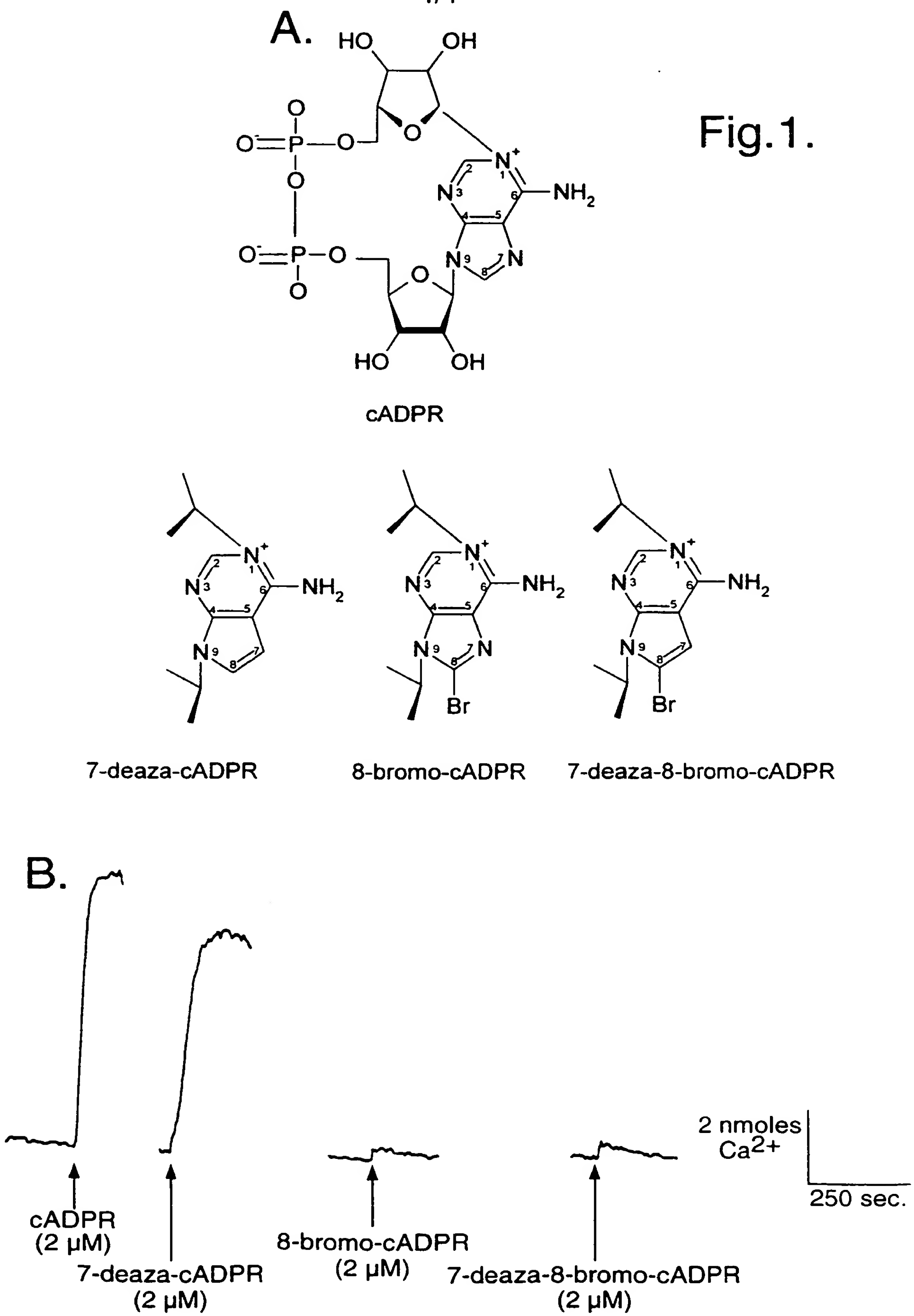

A.
cADPR
7-deaza-cADPR
8-bromo-cADPR
7-deaza-8-bromo-cADPR
B.
2 nmoles Ca2+
250 sec.
cADPR (2 μM)
7-deaza-cADPR (2 μM)
8-bromo-cADPR (2 μM)
7-deaza-8-bromo-cADPR (2 μM)

Fig.2.

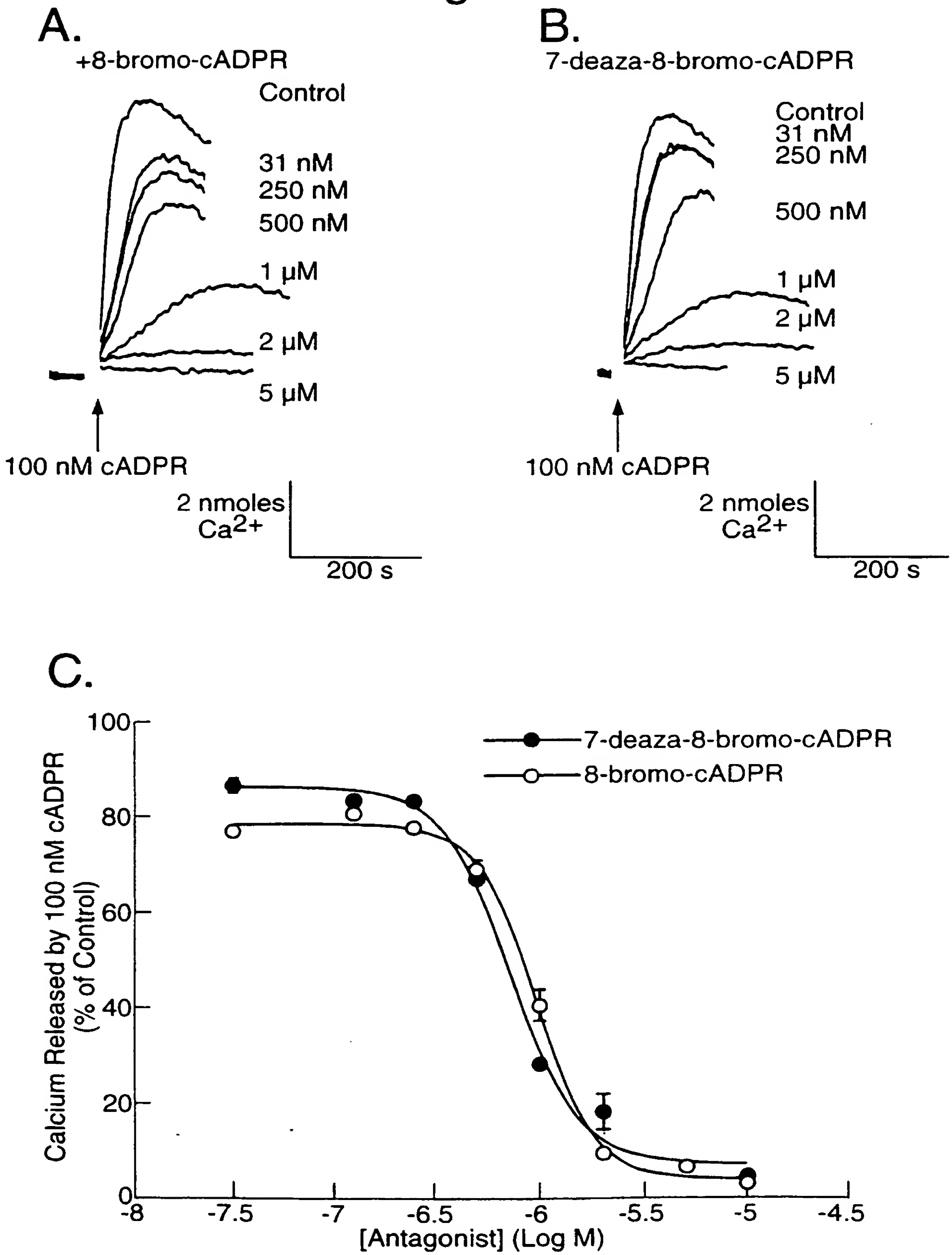

A.
+8-bromo-cADPR
Control
31 nM
250 nM
500 nM
1 μM
2 μM
5 μM
100 nM cADPR
2 nmoles Ca2+
200 s
B.
7-deaza-8-bromo-cADPR
Control
31 nM
250 nM
500 nM
1 μM
2 μM
5 μM
100 nM cADPR
2 nmoles Ca2+
200 s
C.
Calcium Released by 100 nM cADPR (% of Control)
0
20
40
60
80
100
-8
-7.5
-7
-6.5
-6
-5.5
-5
-4.5
[Antagonist] (Log M)
7-deaza-8-bromo-cADPR
8-bromo-cADPR

Fig.3.

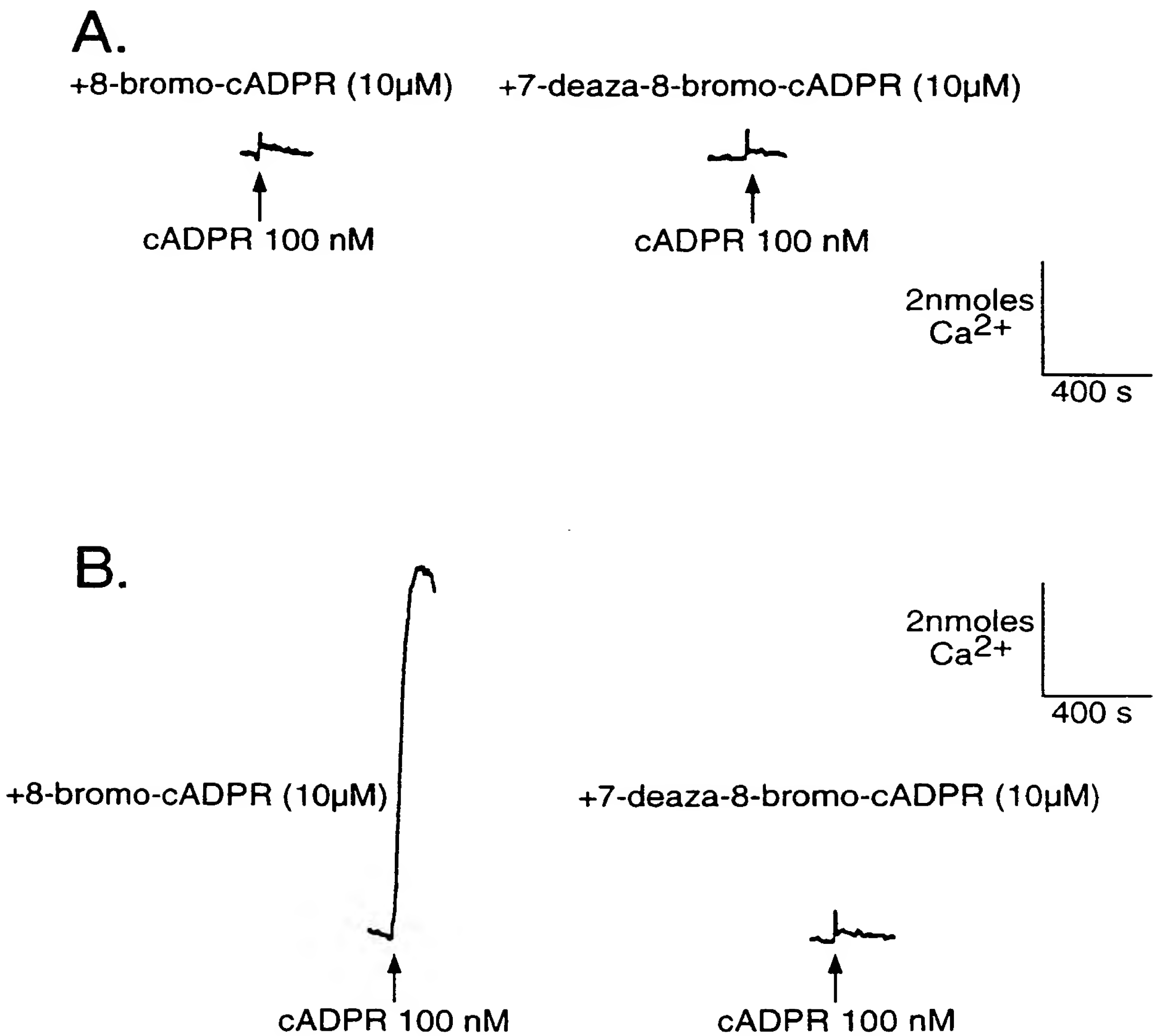
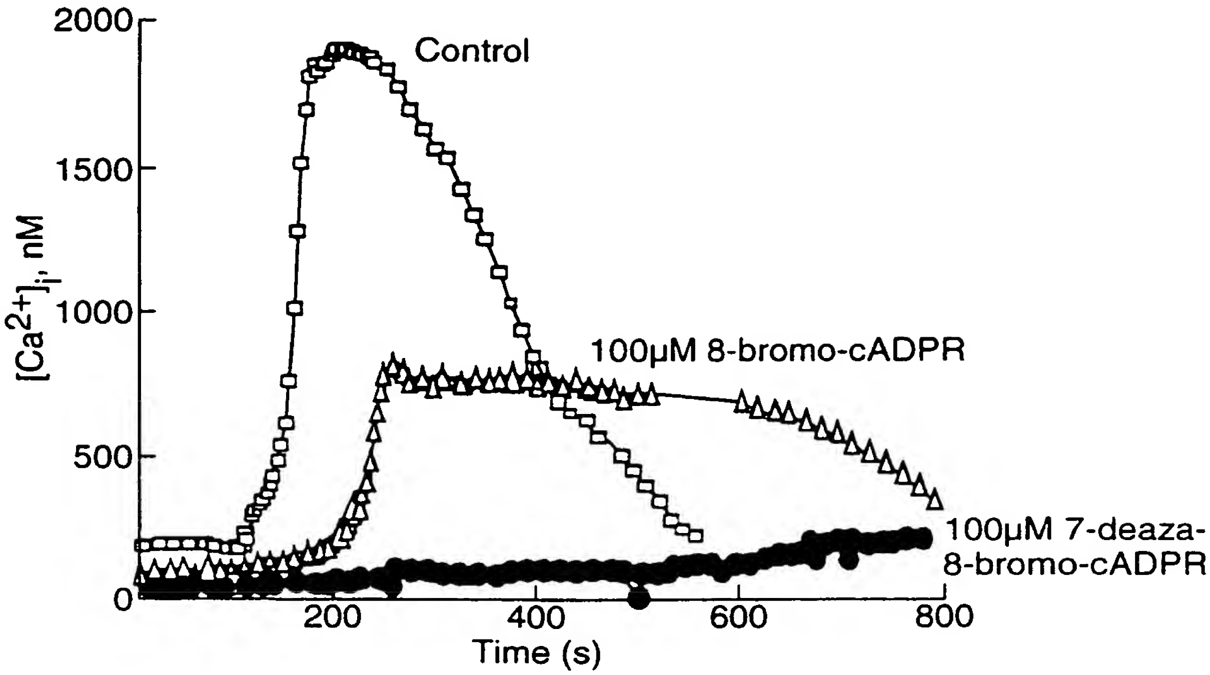
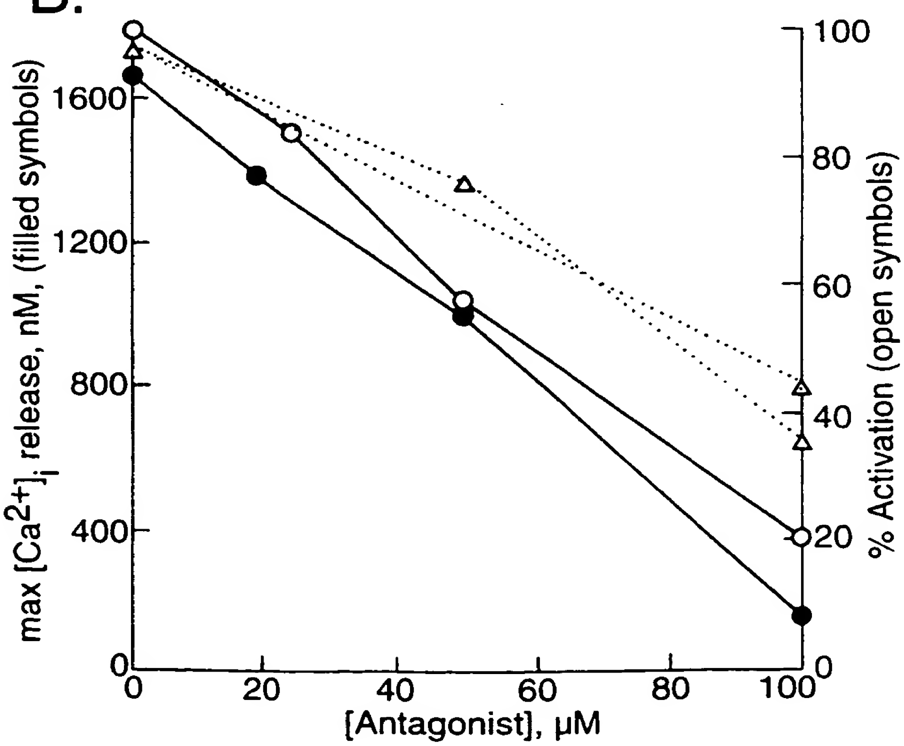

A.
+8-bromo-cADPR (10μM)
+7-deaza-8-bromo-cADPR (10μM)
cADPR 100 nM
cADPR 100 nM
2nmoles Ca2+
400 s
B.
2nmoles Ca2+
400 s
+8-bromo-cADPR (10μM)
+7-deaza-8-bromo-cADPR (10μM)
cADPR 100 nM
cADPR 100 nM

Fig.4.

A.


2000
1500
1000
500
0
[Ca2+]i, nM
200
400
600
800
Time (s)
Control
100μM 8-bromo-cADPR
100μM 7-deaza-
8-bromo-cADPR

B.


1600
1200
800
400
0
max [Ca2+]i release, nM, (filled symbols)
100
80
60
40
20
0
% Activation (open symbols)
0
20
40
60
80
100
[Antagonist], μM

INTE TIONAL SEARCH REPORT

International Application No
PCT/GB 98/00921

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H19/20 C07H21/00 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 486 604 A (WALSETH TIMOTHY F ET AL) 23 January 1996 see abstract; figure 10 ---	1-3
Y	T.F.WALSETH ET AL.: "Synthesis and Characterization of Antagonists of Cyclic-ADP-Ribose-Induced Ca2+ Release." BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1178, no. 3, 1993, pages 235-242, XP002068814 cited in the application see the whole document ---	1-3

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
22 June 1998	09/07/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Scott, J

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	V.C.BAILEY ET AL.: "7-Deaza Cyclic Adenosine 5'-Diphosphate Ribose : First Example of a Ca2+-Mobilizing Partial Agonist Related to Cyclic Adenosine 5'-Diphosphate Ribose." CHEMISTRY AND BIOLOGY, vol. 4, 1997, pages 51-61, XP002068815 cited in the application see the whole document ---	1-3
Y	E.BUTT ET AL.: "Analysis of the Functional Role of cGMP-Dependent Protein Kinase in Intact Human Platelets Using a Specific Activator 8-para-Chlorophenylthio-cGMP." BIOCHEM. PHARMACOL., vol. 43, 1992, pages 2591-2600, XP002068816 cited in the application see the whole document ---	1-3
P,X	V.C.BAILEY ET AL.: "Synthesis of 7-Deaza-8-bromo Cyclic Adenosine 5'-Diphosphate Ribose : the First Hydrolysis Resistant Antagonist at the cADPR Receptor." CHEMICAL COMMUNICATIONS., no. 7, 7 April 1997, ROYAL SOCIETY OF CHEMISTRY GB, pages 695-696, XP002068817 see the whole document -----	1-3

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/00921

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5486604 A	23-01-1996	NONE	